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COLONY SPECIFICITY IN THE ASCIDIAN, *PEROPHORA SAGAMIENSIS*[1]

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Abstract

The presence of colony specificity, *i.e.*, fusion-incompatibility, was revealed in *Perophora sagamiensis*.

When two fusible stolons made a tip-to-side contact, a protrusion appeared from the lateral portion of a stolon. The tests and epidermides of both stolons fused and blood exchange was established. When two nonfusible stolons came into contact, the tip of a stolon became inflated, and the lateral portion of the other stolon protruded with thickened epidermis. After a decrease in the blood stream, cellular parts of both stolons regressed.

Two types of nonfusion were found in *Perophora sagamiensis* and were termed "nonfusion type A" and "nonfusion type B," respectively. In nonfusion type A, two stolons rejected each other without fusion between the tests; while in nonfusion type B, rejection occurred after a transitory fusion of the tests. A given colony showed nonfusion type A to some colonies, and nonfusion type B to some other colonies.

Histological study of the process of nonfusion revealed amoebocytes and lymphocyte-like cells in the contact area.

Introduction

Multicellular invertebrates, from sponges to protochordates, can recognize self and non-self distinctly (see Hildemann *et al.*, 1979, for review). Furthermore, some of them display allogeneic polymorphism and specific memory in transplantation immunity (Cooper, 1970; Hildemann *et al.*, 1980a, b).

Ascidians, a group of the subphylum Protochordata, respond to foreign bodies in various ways. In the body fluid of some solitary ascidians, many humoral substances have been reported, as represented by a nonspecific precipitin to rabbit serum (Cantacuzene, 1913), agglutinins to foreign spermatozoa (Tyler, 1946), agglutinins to mammalian erythrocytes (Fuke and Sugai, 1972), and natural bactericidins (Johnson and Chapman, 1970). Several types of cellular response have also been described. Small particles of dyes (Ivanova-Kazas, 1966; Smith, 1970; Anderson, 1971) and certain bacteria (Thomas, 1931) are phagocytized. Large particles of dyes (Ivanova-Kazas, 1966) and inserted glass fragments (Anderson, 1971) are encapsulated by blood cells. In some solitary ascidians allograft rejection is accompanied by the infiltration of vanadocytes (Anderson, 1971) or by that of lymphocyte-like cells at the interface (Reddy *et al.*, 1975).

Allogeneic recognition in colonial ascidians has been well documented by the study of colony specificity, manifested by fusion-incompatibility between two col-

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onies. Some colonies fuse to form a single mass, but others do not. In *Botryllus primigenus*, the fusibility was shown to be under genetic control (Oka and Watanabe, 1957, 1960, 1967). In all compound ascidians studied so far, contact between fusible colonies results in the union of both the tests and the common vascular systems (if present) of the two colonies. However, there are some differences among species in the mode of rejection. In *Botrylloides simodensis* (Mukai and Watanabe, 1974) and *Perophora japonica* (Koyama and Watanabe, 1981), the tests never fuse between nonfusible colonies. In *Botryllus primigenus* (Oka and Watanabe, 1957) and *Didemnum moseleyi* (Mukai and Watanabe, 1974), rejection always occurs after fusion between the tests of two colonies; whereas in *Botryllus scalaris* rejection occurs after a fusion not only between the tests but also between the test vessels (Saito and Watanabe, 1979). (For the scientific names of *Botryllus scalaris* and *Botrylloides simodensis*, see Saito *et al.*, 1981a, b.)

We have been studying colony specificity in some Japanese species of the Perophoridae. In *Perophora formosana* colony specificity is absent (Mukai and Watanabe, 1974); while colony specificity is present and is expressed in the test surface in *Perophora japonica* (Koyama and Watanabe, 1981). In the present study, we describe the processes of fusion and nonfusion observed in *Perophora sagamiensis*.

Materials and Methods

Several living colonies of *Perophora sagamiensis* were collected from Hatakejima Island in Tanabe Bay, Wakayama, Japan. They were attached to microscopic slides and reared in a bay near the Shimoda Marine Research Center, Shizuoka, Japan. In a colony, respective zooids are connected with one another by vascular stolons from which new individuals arise as small buds. The stolons are sometimes branched and interconnected within a colony, giving a mesh-like appearance.

Experimental procedures were similar to those described in a previous paper (Koyama and Watanabe, 1981); two zooid-stolon systems were tied upon a glass slide, about 1 cm apart and at about right angles to each other, so as to make a contact between the tip of one stolon and the lateral portion of the other stolon. In this paper, the former stolon will be termed "t-stolon" and the latter stolon "l-stolon." Repeated observations were made following the onset of a tip-to-side contact between the two stolons.

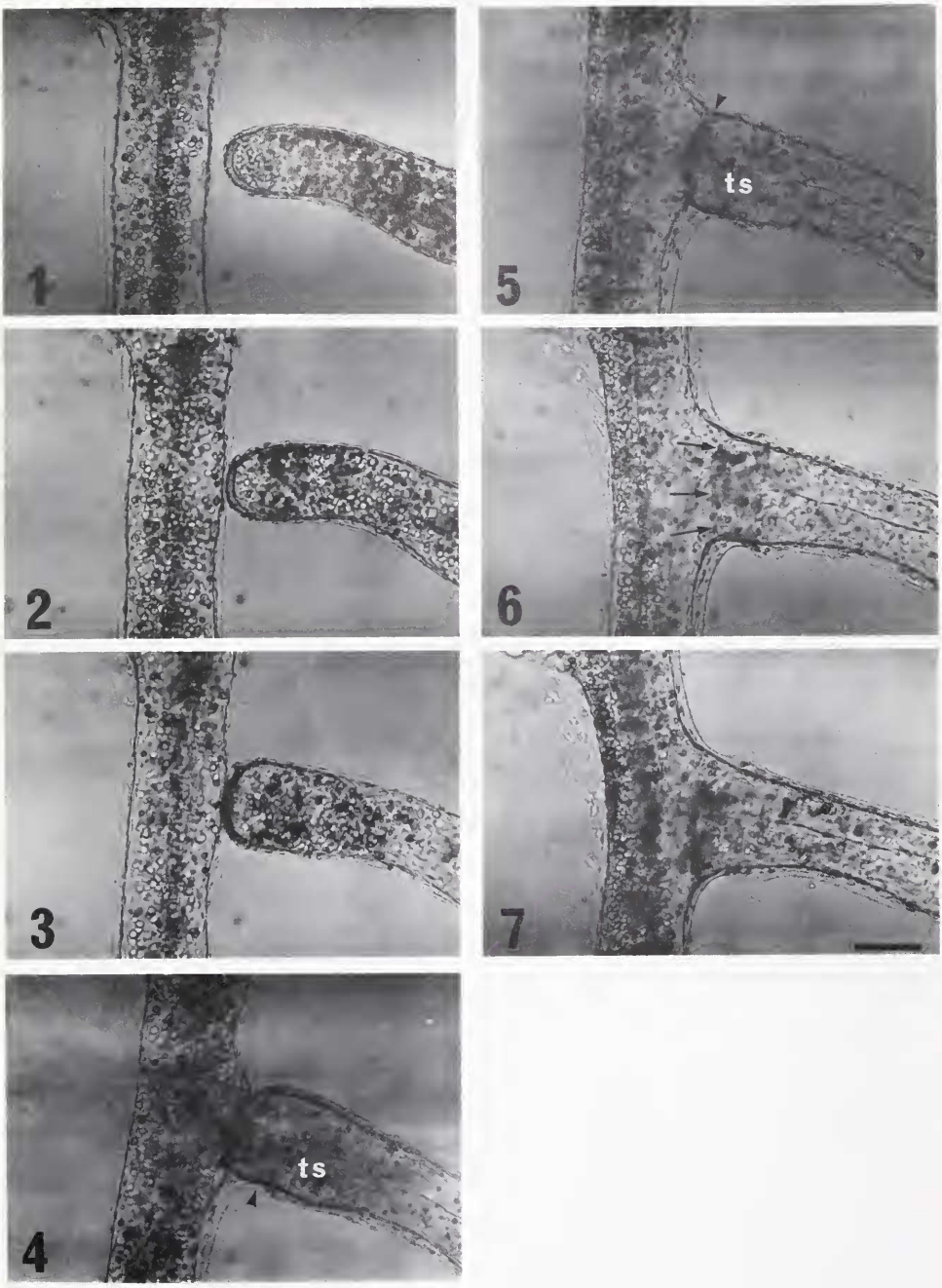
For the histological study, some specimens in the process of nonfusion were fixed in Bouin's solution made in sea water, dehydrated through a butanol series and embedded in paraffin. They were sectioned at 4 μm and stained with Delafield's haematoxylin and eosin-orange G.

Results

The process of fusion

When a tip-to-side contact was made between two stolons derived from a colony, fusion always took place. A typical example of fusion is shown in Figures 1–7.

No change was observed in either stolon prior to contact (Fig. 1). After contact the tip became somewhat flat (Fig. 2). Then, a protrusion appeared in the lateral portion of the l-stolon (Fig. 3). Five hours after contact (Fig. 4), in the t-stolon the tip became inflated and the terminus of the septum (ts) was near the contact area. The protrusion of the lateral portion increased in height, and the test of the l-stolon encroached upon the test of the t-stolon (arrowhead). Six hours after contact



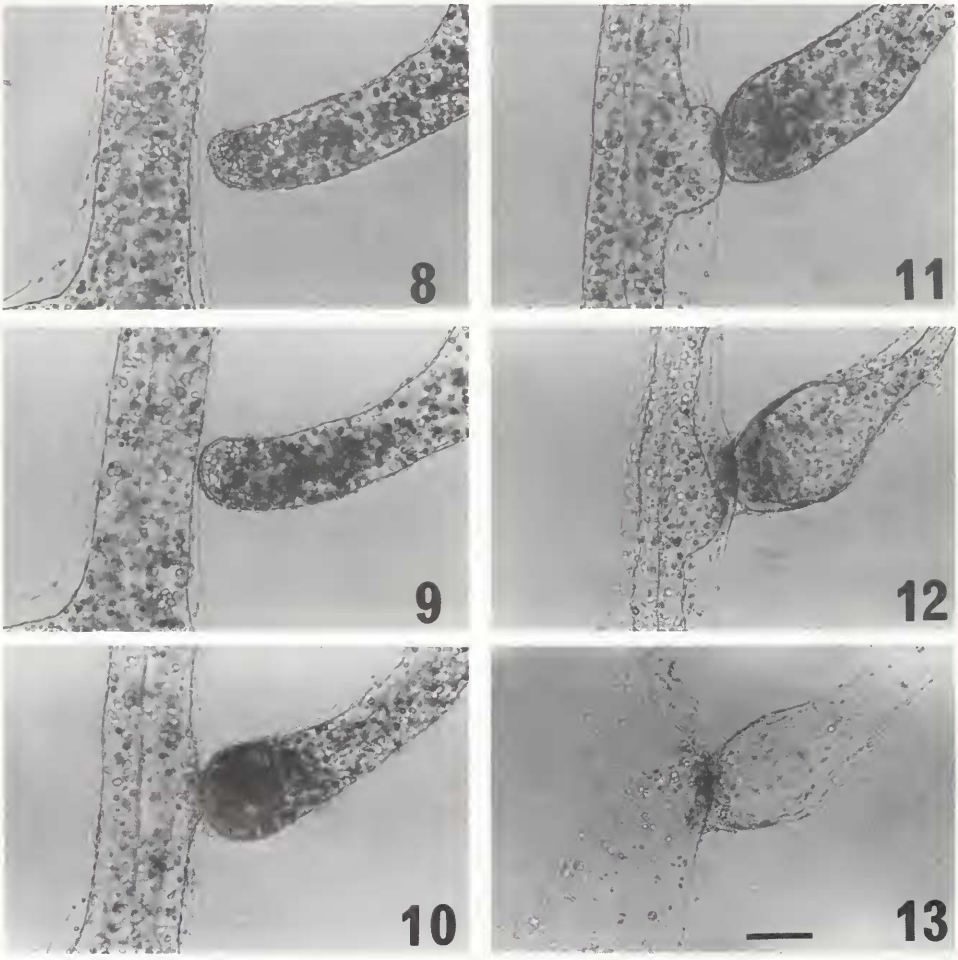
FIGURES 1–7. A typical example of fusion. (Fig. 1): 50 min before contact. (Fig. 2): 16 min after contact. (Fig. 3): 2 h after contact. (Fig. 4): 5 h after contact. The arrowhead indicates the test of the l-stolon encroached upon the test of the t-stolon. ts, terminus of the septum in the t-stolon. (Fig. 5): 6 h after contact. The arrowhead shows the union of epidermis between the two stolons. ts, terminus of the septum in the t-stolon. (Fig. 6): 8 h after contact. The arrows indicates epidermal cells remaining in the anastomosing area. (Fig. 7): 10 h after contact.

All figures are the same magnification; the bar in Figure 7 is 100 μm long.

(Fig. 5), a small amount of blood interchange was established between the two stolons. The epidermides of both stolons were partly connected (arrowhead). In the t-stolon, the terminus of the septum (ts) was almost at the contact area. As fusion proceeded both between the tests and between the epidermides, the remaining epidermal cells (arrows) in the anastomosing area were gradually eliminated (Fig. 6). Then, fusion was completed both in the test and in the epidermis, and the remaining epidermal cells disappeared (Fig. 7).

The process of nonfusion

In *Perophora sagamiensis*, we found two types of nonfusion. One will be termed "nonfusion type A" and the other "nonfusion type B" hereafter. In nonfusion type A, the two stolons reject each other without fusion between their tests; while in nonfusion type B, rejection occurs after a transitory fusion of the tests.



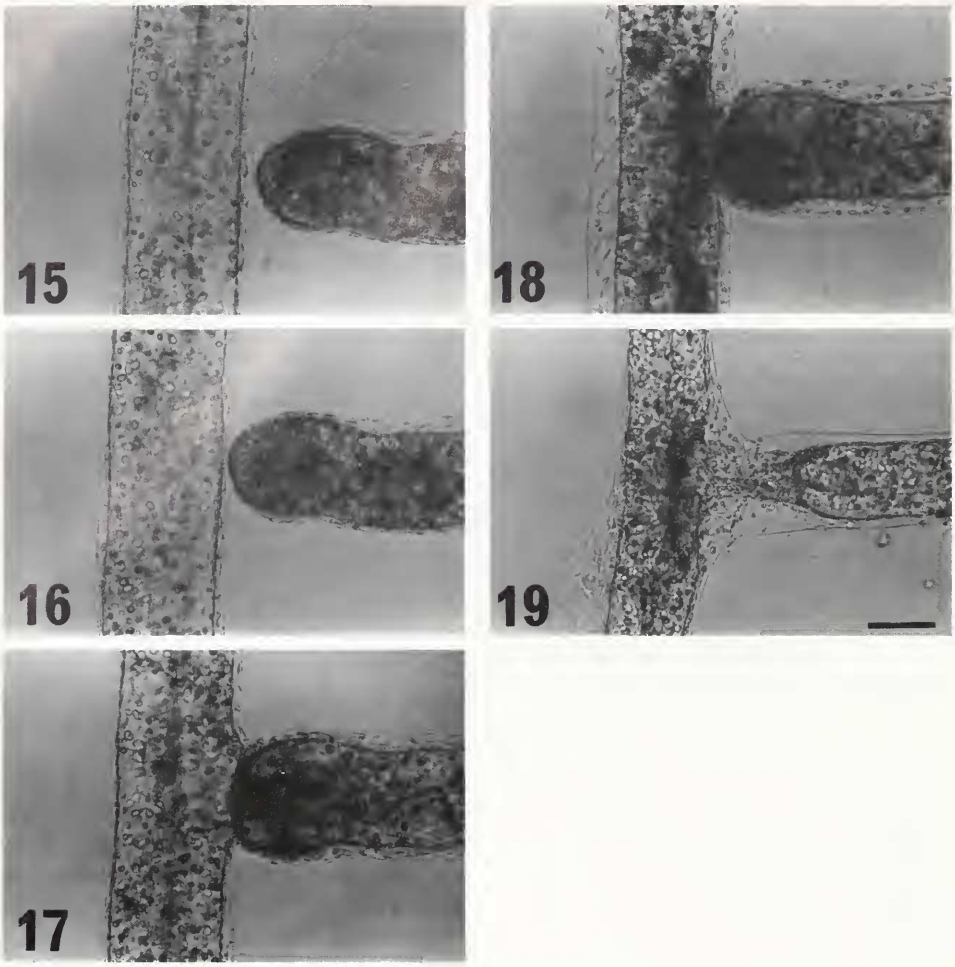
FIGURES 8–13. A typical example of nonfusion type A. (Fig. 8): 45 min before contact. (Fig. 9): Immediately after contact. (Fig. 10): 8 h after contact. (Fig. 11): 24 h after contact. (Fig. 12): 96 h after contact. (Fig. 13): 136 h after contact.

All figures are the same magnification; the bar in Figure 13 is 100 μm long.

FIGURE 14. A histological figure of nonfusion type A, after detachment of the two stolons. el, external layers of the test; epl, thickened epidermal cells of the l-stolon; ept, epidermal cells of the t-stolon. Scale bar = 50 μm.

Figures 8–13 show a typical example of nonfusion type A. The t-stolon was growing to the l-stolon without a noticeable change (Fig. 8). Then, a contact was made between the two stolons (Fig. 9). After contact, a protrusion appeared in the lateral portion (Fig. 10). The epidermis of the protrusion thickened. Twenty-four hours after contact (Fig. 11), the dilatation of the tip and the protrusion of the lateral portion were at maximum, but attenuation of the stolon had already begun. Later, distal parts of both stolons became thinner and their blood stream decreased (Fig. 12). After that, the cellular parts of both stolons regressed (Fig. 13), leaving empty test tubes in the original place. Disintegration of interacting stolons was not observed, unlike in the case of nonfusion of *Botryllus primigenus* (Oka and Watanabe, 1957).

Throughout the reaction of nonfusion type A described above, a clear demarcation line was obvious between the two stolons. Furthermore, at any stage of the reaction, the two stolons could readily be separated from each other by pulling them without causing noticeable damage. Even after two stolons were detached, the external layers of the test could be recognized (Fig. 14). From these observations, it may be concluded that no union of test matrices is established in nonfusion type A.



FIGURES 15–19. Serial photomicrographs of nonfusion type B. (Fig. 15): 15 min before contact. (Fig. 16): Immediately after contact. (Fig. 17): 3 h after contact. (Fig. 18): 4 h after contact. (Fig. 19): 28 h after contact.

All figures are the same magnification; the bar in Figure 19 is 100 μm long.

Figures 15–19 are serial photomicrographs of nonfusion type B. When the t-stolon was growing toward the l-stolon, no specific change was observed in either of them (Fig. 15). Soon, the two stolons came into contact (Fig. 16). Then, a protrusion appeared in the lateral portion (Fig. 17). After a while, the two stolons dwindled and their blood stream decreased (Fig. 18). Finally, the cellular parts of the two stolons detached from each other (Fig. 19) and regressed.

In the later stages of nonfusion type B, the demarcation line between the two stolons became obscure and the test tubes of the two stolons were no longer separable. Histologically, no external layers of the test could be recognized between the epidermal cells of the two stolons (Fig. 20). Moreover, in the case shown in Figure 20, there was a bridge of epidermal cells connecting the two stolons. These results may be accepted as evidence for the fusion of the tests in nonfusion type B.

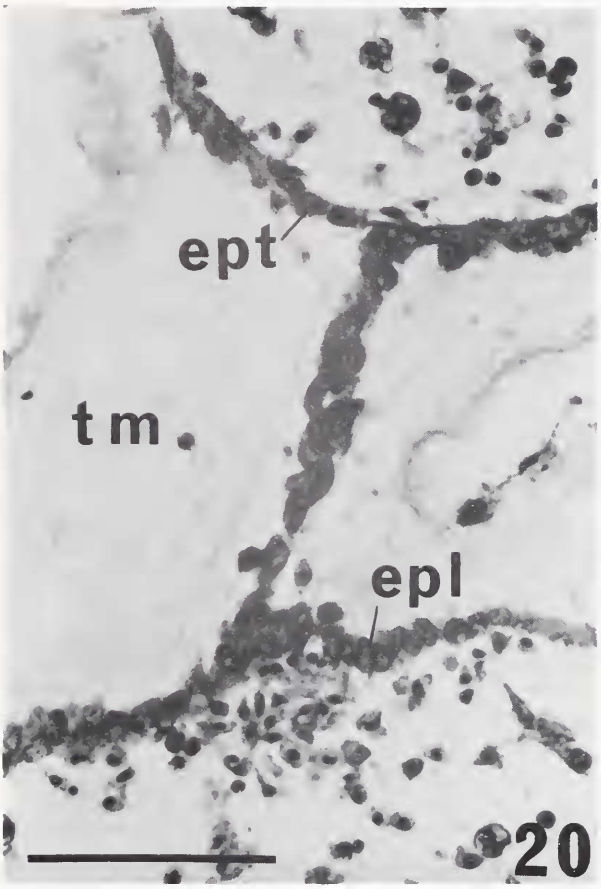


FIGURE 20. A histological figure of nonfusion type B, after detachment of the two stolons. Many amoebocytes were observed in the lumen of the l-stolon. epl, epidermal cells of the l-stolon; ept, epidermal cells of the t-stolon; tm, test matrix. Scale bar = 50 μm.

Fusibility between different colonies

The two types of nonfusion did not take place at random. A particular combination of incompatible colonies always exhibited only one type of nonfusion.

We first tested the fusibility of a certain colony to twelve other colonies. Of the twelve combinations, four produced fusions, five produced nonfusions of type A, and the remaining three produced nonfusions of type B. Next, six colonies were selected and fusibility among them was tested in all possible binary combinations. The results are shown in Table I. For example, Col. 1 fused with Cols. 2, 3 and 4. However, in the combinations among Cols. 2, 3 and 4, only Col. 2 and Col. 4 fused and the other two combinations showed nonfusion type B. When Col. 5 was combined with Col. 1, 2, 3 or 4, nonfusion type B occurred. In every combination with Col. 6, nonfusion type A took place.

Histological observations of nonfusion

Histologically, in either type of nonfusion, two kinds of blood cell were often observed near or on the epidermal cells of the contact area. They were amoebocytes

TABLE I

The fusibility between different colonies taken from nature.

	Col. 1	Col. 2	Col. 3	Col. 4	Col. 5	Col. 6
Col. 1	f	f	f	f	nf B	nf A
Col. 2	f	f	nf B	f	nf B	nf A
Col. 3	f	nf B	f	nf B	nf B	nf A
Col. 4	f	f	nf B	f	nf B	nf A
Col. 5	nf B	nf B	nf B	nf B	f	nf A
Col. 6	nf A	nf A	nf A	nf A	nf A	f

f = fusion, nf A = nonfusion type A, nf B = nonfusion type B.

and lymphocyte-like cells (Figs. 14, 20, and 21). They were observed from four hours after contact and even after detachment of the stolons. The amoebocytes had a distinct nucleus and clear cytoplasm. They were spherical, flattened, or irregular in shape. The nearly spherical lymphocyte-like cells, being 3–4 μm in diameter, had a prominent nucleus and a small amount of basophilic cytoplasm. Their nuclei were round or pear-shaped with a few clusters of chromatin.

DISCUSSION

These results demonstrated that colony specificity is present in *Perophora sagamiensis*.

The processes of fusion and nonfusion type A in this species are similar to those of fusion and nonfusion, respectively, observed in *Perophora japonica* (Koyama

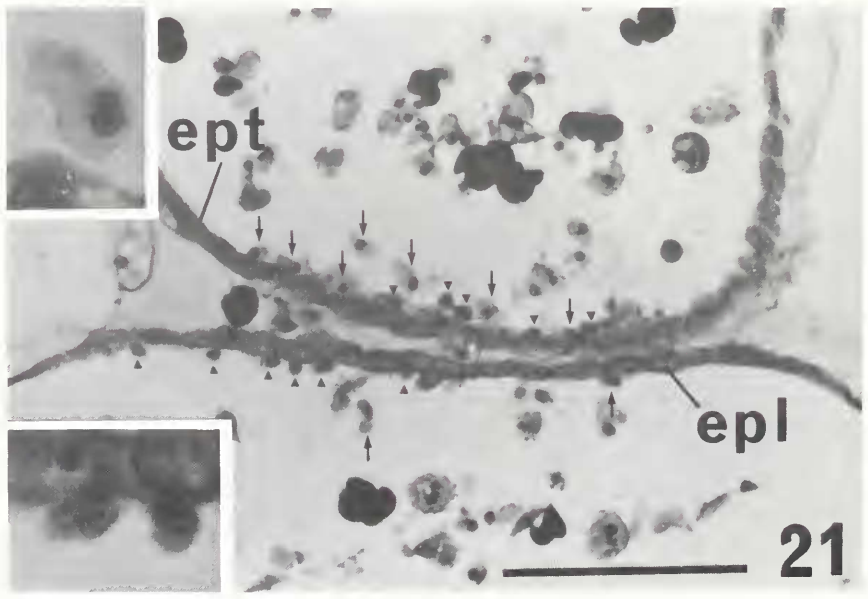


FIGURE 21. Amoebocytes (arrows) and lymphocyte-like cells (arrowheads) near and on the epidermal cells in the contact area in nonfusion type B. 4 h after contact. epl, thickened epidermal cells of the l-stolon; ept, epidermal cells of the t-stolon. Scale bar = 50 μm. Upper inset, a magnified amoebocyte. Lower inset, two magnified lymphocyte-like cells on the epidermal cells.

and Watanabe, 1981). From the present and previous works (Mukai and Watanabe, 1974; Koyama and Watanabe, 1981), the three species of Japanese Perophoridae can be divided into two groups: one without colony specificity and the other having it. *Perophora formosana* belongs to the former, and *P. japonica* and *P. sagamiensis* to the latter group. From the observations of the zooid structure, the way of reproduction and the morphology of blood cells, *Perophora formosana* can be clearly distinguished from the other two species (Tokioka, 1953; and our unpublished data).

The results of the present and the previous fusion experiments in several colonial ascidians are summarized in Table II. In the reaction resulting from contact between natural surfaces of two colonies in respective species, three cases can be distinguished. One case is fusion, in which complete fusion both in the test and in the test vessels is established between the two colonies; another case is rejection, in which some physiological antagonism is seen between the two colonies; the last case in which no particular reaction can be observed is referred to as indifference. Colonial ascidians so far studied can be classified into two major groups. In one group, to which *Polycitor mutabilis* and *Perophora formosana* belong, colony specificity is absent: when two colonies come into contact, indifference occurs between natural surfaces, whereas fusion takes place between cut surfaces, regardless of their origin. In the other group colony specificity is present. Discrimination of self and non-self is made between two colonies, resulting in fusion, rejection, or indifference. This group consists of several subgroups. In the first subgroup, discrimination occurs before fusion between the tests of two colonies. *Botrylloides simodensis* and *Perophora japonica* belong to this subgroup. In the second subgroup, to which *Botryllus primigenus* and *Didemnum moseleyi* belong, discrimination

TABLE II

Summarizing representation of fusion experiments. The "discrimination points" in the text are shown by braces.

Species	Test	Test (blood) vessel	Colony	Authors
Polycitor mutabilis	Nonfusion		Indifference	Oka and Usui, 1944
Perophora formosana	Nonfusion		Indifference	Mukai and Watanabe, 1974
Botrylloides simodensis	Fusion	Fusion	Fusion	Mukai and Watanabe, 1974
	Nonfusion		Indifference	
Perophora japonica	Fusion	Fusion	Fusion	Koyama and Watanabe, 1981
	Nonfusion		Rejection	
Perophora sagamiensis	Fusion	Fusion	Fusion	
		Nonfusion	Rejection (Type B)	
	Nonfusion		Rejection (Type A)	
Botryllus primigenus	Fusion	Fusion	Fusion	Oka and Watanabe, 1957
		Nonfusion	Rejection	
Didemnum moseleyi	Fusion		Fusion	Mukai and Watanabe, 1974
			Rejection	
Botryllus scalaris	Fusion	Fusion	Fusion	Saito and Watanabe, 1979
			Rejection	

takes place after fusion between the tests but before fusion between the test vessels (if present). In the third subgroup discrimination occurs after fusion between the test vessels of two colonies. *Botryllus scalaris* belongs to this subgroup. In all species mentioned above, there is a single discrimination point. In *Perophora sagamiensis*, there are two discrimination points, *i.e.*, before and after fusion between the tests. Therefore, this species has two features, one shared with the first subgroup and the other with the second subgroup.

As seen in Table I, a given colony will show nonfusion type A to some colonies and show nonfusion type B to some other colonies. This may suggest that the fusibility of the test and that of the blood vessel are controlled separately. Type B nonfusible colonies seem to share only the fusibility of the test, and type A nonfusible colonies do not. Detailed genetic analysis is desirable to clarify the controlling factors of fusibility in this species.

Histological study of the process of nonfusion revealed the presence of amoebocytes and lymphocyte-like cells near or on the epidermal cells in the contact area. These two types of blood cell were observed to behave likewise during the process of fusion (unpublished data). It is tempting to speculate that they might participate in the recognition of self and non-self. In *Ciona intestinalis*, infiltration of lymphocyte-like cells accompanied by allograft rejection was observed and their roles in allogeneic recognition and rejection have been discussed (Reddy *et al.*, 1975). Much more study is needed, however, to elucidate the significance of these blood cells in the processes of fusion and nonfusion.

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